

Integration of Ruminal Metabolism in Dairy Cattle^{1,2}

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ABSTRACT

An important objective is to identify nutrients or dietary factors that are most critical for advancing our knowledge of, and improving our ability to predict, milk protein production. The Dairy NRC (2001) model is sensitive to prediction of microbial protein synthesis, which is among the most important component of models integrating requirement and corresponding supply of metabolizable protein or amino acids. There are a variety of important considerations when assessing appropriate use of microbial marker methodology. Statistical formulas and examples are included to document and explain limitations in using a calibration equation from a source publication to predict duodenal flow of purine bases from measured urinary purine derivatives in a future study, and an improved approach was derived. Sources of specific carbohydrate rumen-degraded protein components probably explain microbial interactions and differences among studies. Changes in microbial populations might explain the variation in ruminal outflow of biohydrogenation intermediates that modify milk fat secretion. Finally, microbial protein synthesis can be better integrated with the production of volatile fatty acids, which do not necessarily reflect volatile fatty acid molar proportions in the rumen. The gut and splanchnic tissues metabolize varying amounts of volatile fatty acids, and propionate has important hormonal responses influencing milk protein percentage. Integration of ruminal metabolism with that in the mammary and peripheral tissues can be improved to increase the efficiency of conversion of dietary nutrients into milk components for more efficient milk production with decreased environmental impact.

Key words: microbial protein synthesis, milk protein, rumen carbohydrate degradation, volatile fatty acid metabolism

INTRODUCTION

The first objective of the USDA Regional Project NC-1009, entitled *Metabolic Relationships in Supply of Nutrients for Lactating Dairy Cows*, is to quantitatively evaluate chemical and physical properties of protein and energy sources that determine the availability of nutrients critical to milk protein secretion in lactating dairy cows. In particular, efforts are focused toward which nutrients or feed processing procedures are most critical for advancements in knowledge in both biology (nutritionally relevant) and statistics (parameters that are more likely to improve prediction). From a mechanistic (biological) standpoint, milk protein production is a function of the supply of limiting AA from endogenous, microbial, and undegraded feed proteins (NRC, 2001) but also is influenced by hormonal changes mediated by ruminal events (e.g., increased propionate production increases insulin secretion; Brockman, 1993). Improved accuracy of equations is an obvious goal to improve predictability of factors influencing milk protein secretion; however, statistical procedures often only validate precision, whereas inferences have sometimes been made inappropriately toward accuracy of relationships among variables. Yet, when the same equation or similar relationships are used for multiple predictions, improved precision also helps reduce amplification of error and thereby could improve accuracy in an entire system. Thus, objectives of this review are 1) to highlight important findings and future perspectives regarding some selected mechanistic aspects of rumen function, and 2) to discuss important issues limiting our ability to quantitatively integrate those results to improve the accuracy and precision of prediction of supply of AA and VFA for whole-animal metabolism systems.

Variables That Influence Milk Protein Production

Integration of Ruminal Metabolism to Milk Protein Production. The common mission linking all ob-

Received August 16, 2005.

Accepted December 23, 2005.

¹Presented at the ADSA/ASAS/CSAS Joint Annual Meeting, Cincinnati, OH, July 2005.

²Salaries and research support were provided by state and federal funds appropriated to the Ohio Agricultural Research and Development Center, the Ohio State University; Manuscript No. 25-05AS.

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jectives of NC-1009 is to improve the efficiency of milk protein production. Several members of this committee recently reviewed aspects related to this topic (Bach et al., 2005; Firkins and Reynolds, 2005; Hristov and Jouany, 2005; Ipharraguerre and Clark, 2005), and 4 members of NC-1009 were on the NRC (2001) committee. Thus, we refer readers to these publications and will limit our discussion to recent information related to improving either the supply of MP and metabolizable AA (i.e., absorbed from the small intestine) or to improving our ability to maintain MP supply with a lower input of dietary CP.

In two landmark reviews, Clark et al. (1992) and Stern et al. (1994) elaborated on how efficiency of microbial protein synthesis (E_{MPS}), AA supply to the small intestine, and the intestinal digestibility of RUP influence milk protein production. Since that time, progress has been made, but integration of multiple studies is needed to use past reports to further improve efficiency of dietary protein use. In a comprehensive meta-analysis of experiments studying substitution of various protein sources (Ipharraguerre and Clark, 2005), microbial protein flow to the duodenum was depressed on average by 7%, partially negating the benefit of the RUP source to increase overall supply of MP. However, provision of the RUP source increased the duodenal flow of either lysine or methionine enough to compensate for the depressed microbial protein synthesis (MPS), on average, even though it did not do so in all individual studies.

Although indirectly accounting for variation among studies by scaling protein source means relative to their respective controls within trials, the approach of Ipharraguerre and Clark (2005) probably did not differentiate the total effect of the protein source treatment from the effects of the protein source per se. For example, replacement of protein sources higher in RUP for those with more RDP (typically solvent soybean meal) often depressed DMI, either numerically or significantly. If RDP were more limited for the test protein source than for the control, a response variable would include the effects of this limitation. The design of many experiments to substitute large amounts of one protein source for another while maintaining constant CP explains in part why the effect of various RUP sources on milk protein production was disappointing (neutral or even negative) in their analysis. When trying to incorporate effects of microbial protein and RUP supplies, the authors found that there was a strong relationship between total nonammonia N flow to the duodenum and N intake (both of which include DMI as a driving variable). Therefore, if inferences are to be made on how protein supplementation affects metabolizable AA supply, with their being independent from changes in MPS or DMI (assuming these would not change if RDP was

adequate), the previous approach integrating the results of past studies would be difficult to exploit for prediction of future responses when RDP would not be deficient.

When using various protein sources as class (discontinuous) variables, authors can include in the model other sources of variation as continuous variables that are common among studies, as demonstrated by Firkins et al. (2001) for grain sources. Therefore, the means for the class variables (i.e., protein sources) would be adjusted to the average effect of the continuous variables remaining in the model while still being scaled against a common control within study (e.g., coding the solvent soybean meal control as a class variable that is separate from the higher RUP protein sources). Assuming the data are reasonably well balanced (e.g., some studies with the same protein source comparisons would be deficient in RDP and some would not), the class variables could be adjusted for the effect of RDP (or any continuous variable in the model), and least squares means could be compared at the average RDP concentration from the total data set. In other words, assuming a balanced data set, these could be compared as if all other variables were equalized. We caution that researchers need to be aware that having both continuous variables plus class variables in the same model requires some interpretation, as described in Table 5 from Firkins et al. (2001). We also highly recommend using the Mixed procedure of SAS (SAS Institute, Inc., Cary, NC) because the GLM procedure requires manual adjustment of results to the average effect of trial (Oldick et al., 1999), and we note cases of biased models reported in the literature when this appears not to have been done. Finally, graphic depiction of fits using unadjusted data are not nearly as meaningful as those when the data are adjusted to the random effect of trial before graphing (St-Pierre, 2001a).

A good example of how statistical procedures can be enhanced by consideration of interrelationships among dependent variables can be illustrated from the NRC (2001) equation for milk protein production (kg/d):

$$-1.57 + (0.0275)(DMI) + (0.223)(RDP) \\ - (0.0091)(RDP^2) + (0.041)(RUP)$$

in which DMI units are kg/d and RDP and RUP are percentage of DM. Although the r^2 was 0.51, which is fairly robust for this approach, the effects of experiment and unequal variance among measurements within experiments were not considered, and such effects have tremendous impact on the resulting equation (Oldick et al., 1999; Firkins et al., 2001; Hristov et al., 2004b). Therefore, we reconstructed the entire NRC data set

(206 treatment means from 38 studies) and verified that the resulting regression equation was virtually identical to that above. As in a previous meta-analysis (Firkins et al., 2001), we then included the effects of experiment and weighted the observations relative to the reciprocal of each study's SEM, except in a few cases in which the SEM was bounded at one-half the average SEM among all studies to prevent overweighting of data from individual studies. Data were subjected to backward elimination multiple regression with those same 3 variables in the previous equation and all possible squared terms (including interactions). In all cases, the random effect of experiment was $P < 0.0001$. The resulting model for milk protein production (kg/d) =

$$-0.436 + (0.0590)(\text{DMI}) + (0.0569)(\text{RDP}) \\ + (0.0202)(\text{RUP}) - (0.00255)(\text{RDP} \times \text{DMI}) \quad [1]$$

In this model, the root mean square error (**RMSE**) after adjusting for the effect of study was 0.047, and all terms were $P < 0.01$ except the intercept ($P = 0.08$). The SE were 0.241, 0.0110, 0.0207, 0.0033, and 0.00098, respectively. In agreement with Hristov et al. (2004b), DMI had an important influence on milk protein production. Interestingly, at the mean DMI of 20.6 kg/d, the effect of RDP is relatively flat. As DMI decreases, increasing RDP is associated with increasing milk yield in a diminishing response until the maximum of 22.3 kg/d. As DMI increases beyond 22.3 kg/d, RDP would be predicted to have an increasingly negative response, which is in contrast with well-known nutrition principles in which increasing RDP can directly stimulate DMI through effects on ruminal fiber digestibility and decreased fill restriction (Allen, 2000).

This empirically derived regression might lead to confusing biological inferences. Using the same data and the same backward elimination multiple regression procedure, though, DMI was related linearly to RDP:

$$\text{DMI, kg/d} = 19.3 + (0.141)(\text{RDP, \% of DM}) \quad [2]$$

(RMSE = 1.01; SE = 0.1 and 0.072, respectively). Based on the slope, this model has approximately one-fourth of the impact compared with the NRC (2001) model [$\text{DMI} = 14.4 + (0.58)(\text{RDP})$]. To integrate the concept that RDP has a marginal effect on DMI, the right-hand side of [2] was substituted for the DMI terms in [1], as rationalized by St-Pierre (2001b). After substitution and consolidation of terms, we obtained the resulting equation:

$$\text{Milk protein, kg/d} = 0.703 + (0.0160)(\text{RDP}) \\ - (0.000360)(\text{RDP}^2) + (0.0202)(\text{RUP}) \quad [3]$$

In [1], the interaction of RDP with DMI minimized the effect of RDP and even implied negative effects; however, in [3], accounting for the indirect effect of increasing RDP to increase DMI, the result is simplified and more consistent with our expectations. The regression coefficient for RUP indicates that each incremental percentage unit increase in RUP would be expected to increase milk protein production by a modest 0.0202 kg/d, which is about half of what would be expected using the NRC model described above (regression coefficient of 0.041). Interested readers are directed to the recent meta-analyses of Hristov et al. (2004b, 2005b) for a wider evaluation of dietary variables related to DMI and milk protein yield.

Considerable variation remains in our ability to balance diets to meet demands for metabolizable AA supply (NRC, 2001; Schwab et al., 2005). For example, despite increased MP supply through supplementation of soybean protein sources with increased RUP without any detrimental effects on MPS, the supply of methionine was not increased and duodenal lysine supply only tended ($P < 0.06$) to be increased (Ipharraguerre et al., 2005a); the latter result indicates the challenge with documenting changes in metabolizable AA supply with low numbers of animals. In another recent study, despite increasing duodenal supply of both methionine and lysine with increasing dietary CP, the percentage of CP could be decreased to improve the efficiency of conversion of dietary protein into milk protein (Ipharraguerre et al., 2005b). However, Socha et al. (2005) discussed how prepartum supplementation of rumen-protected lysine or rumen-protected methionine in the prepartum period allowed lower dietary protein (16%) in the postpartum period to provide comparable results to higher protein (18.5%). Such results seem to indicate that short-term experimental periods influence milk protein responses from some studies. Interestingly, rumen-protected methionine did not increase milk protein production even though metabolizable methionine was estimated by the NRC (2001) model to be more limiting than metabolizable lysine. Digestibility of the intestinal lysine supply and other sources of variation associated with postabsorptive processes need further attention to prioritize conditions when metabolizable AA from RUP or rumen-protected AA will have a greater likelihood of success.

There are various methods to estimate RDP and RUP, and all have important sources of error, including issues related to accuracy and precision of estimation of passage rate (Firkins et al., 1998) and digestion rates in vitro (Broderick et al., 2004) and in situ (Bach et al., 1998). Future researchers should be aware that pulse-dosing a nonphysiological amount of some AA or related product can change its degradation rate (Bach et al.,

2005) and likely underestimates its disappearance kinetics compared with an actual feeding condition in which the pool of that product would not have been perturbed (Firkins and Reynolds, 2005). This point was corroborated in a recent study (Robinson et al., 2005). Despite these limitations, the NRC (2001) chose to use an in situ model because of the large amount of data available. Many members of NC-1009 have significantly improved laboratory measurements for evaluation of protein degradability and intestinal digestibility (Stern et al., 1997), and this type of research is needed to standardize and improve our ability to estimate the supply of MP (and eventually extended to metabolizable AA) more accurately and precisely.

Contribution of Microbial Protein to MP Systems

The AA of microbial origin are well balanced with respect to requirements of absorbed AA and are assumed to be relatively constant in AA profile (NRC, 2001), although microbial subpopulations probably vary significantly in AA proportions (Volden et al., 1999; Rodríguez-Prado et al., 2004) and the AA composition of bacteria could change with growth rate (Bach et al., 2005). Moreover, the complementary AA in RUP must be digestible in the small intestine. Consequently, a metabolizable AA system needs further research.

The NRC (2001) plotted milk protein responses against lysine and methionine supplies, calculated as percentages of MP supply. Although being a necessary format because of limitations of the data set, many subsequent users have inferred requirements of metabolizable lysine and methionine from the breakpoints in these dose-response graphs. This practice allows the metabolizable AA supply to be increased either by increasing AA flows to the duodenum or by independently decreasing the supply of MP. That is, one could conceivably increase methionine as a percentage of MP by changing dietary conditions such that MP is decreased to a greater degree than methionine is decreased, perhaps explaining some of the variability shown in the response curves (lower precision) but also potentially explaining why expected responses (accuracy) are sometimes not realized (e.g., as in Socha et al., 2005).

When expressed as a percentage of MP, the prediction of metabolizable AA requirement is even more sensitive to errors associated with MPS prediction. If MPS is under- or overpredicted, both lysine and MP supply could be under- or overestimated proportionately, and the effect on accuracy might be partially buffered. However, as the error in MPS prediction increases, making a ratio of AA:MP could compound this error and decrease precision. The requirement of RDP is calculated as microbial protein flow/0.85, and the requirement of digest-

ible RUP is calculated after the predicted digestible microbial protein supply is subtracted from the factorial requirements for MP. Such an enormous impact of MPS calculations for both supply and requirement functions disposes the model to be susceptible to amplification of errors from using predictions for predictions and even more from using ratios. Therefore, real progress for matching supply of MP with requirements depends heavily on improved procedures for measuring and predicting microbial N flow to the duodenum (Firkins and Reynolds, 2005).

Even in more mechanistic models (Dijkstra et al., 1998a), improved quantification of MPS can help provide data for sensitivity analyses and other evaluations or, in some cases, by direct parameterization. Increasing dietary starch can increase both the production of protozoa and the protozoal-mediated recycling of microbial matter (Dijkstra et al., 1998b). Most other models either ignore protozoal-mediated recycling or consider it relatively constant (Dijkstra et al., 1998a). Yet, despite its potential importance for direct parameterization or for challenges of models, methods to quantify protozoal N have received little attention until recently (Sylvester et al., 2005). Also, models often partition bacteria into 2 pools: those that degrade fiber, which typically attach to the particulate phase, and those that degrade nonstructural carbohydrate (NSC), which often are considered to be nonadherent. These models often are based on the ruminal pool sizes, but such data are rarely reported, and such data might help in future meta-analyses evaluating MP systems.

Considerations for Measuring and Predicting Microbial N Flow

Predicted Flow of Microbial N from NRC (2001). Many researchers are comparing their within-study measurements of MPS to those predicted by formulas either for comparison or for validation purposes. The NRC (2001) prediction of microbial protein appears to be robust over a variety of conditions (St-Pierre, 2003), as would be expected because it was empirically derived from a compilation of numerous individual experiments, although it might not be accurate for each specific comparison. Reynal and Broderick (2005) compared microbial N flows to the omasum measured using ¹⁵N and those predicted from NRC (2001); the latter predictions were much lower than those actually measured. However, high-moisture corn was fed at 32% of the diet DM and the treatments exchanged large amounts of soybean meal plus urea for a high RUP soybean meal source (without urea), thus likely prioritizing peptide concentration as a limitation for MPS (see later discussion). In addition, the NRC (2001) ap-

proach was designed to provide an average relationship based on total digestible nutrients from experiments primarily with ground, shelled corn (i.e., largely ignoring differences in site of starch digestion when using high-moisture corn).

Predicted Flow of Microbial N using Urinary Purine Derivatives. In contrast with predicting MPS based on dietary characteristics (such as total digestible nutrients or other more mechanistic inputs), a growing number of researchers are using purine derivatives (PD) excreted in the urine to avoid some of the difficulties associated with traditional measurement of microbial N flow such as low statistical power (Titgemeyer, 1997) or cannulation challenges (Harmon and Richards, 1997). In a review of this approach, Shingfield (2000) documented various sources of error.

In efforts to overcome these problems, researchers have infused known amounts of RNA into the duodenum. Such studies are useful to validate linearity of urinary excretion of PD derived from absorbed purine bases (PB) (Vagnoni et al., 1997) and can be combined with ^{15}N enrichment of PB to estimate the amount of endogenous PD that is produced from normal tissue turnover and degradation of nucleic acids and excreted in the urine (González-Ronquillo et al., 2003). However, the uncertainty of the true intercept from these infusion studies still limits the accuracy of resulting equations for prediction purposes for future studies. Even though cows in early (wk 10) vs. late (wk 33) lactation had intercepts of 104 and 84 mmol/d of PD, González-Ronquillo et al. (2003) forced a common mechanistically derived intercept of 58.9 mmol/d for 2 separate prediction equations (each with different linear regression coefficients). However, the intentional forcing of an intercept to another value that is statistically different will decrease accuracy as predictions deviate further from the mean. Although intentional infusion of known amounts of PB into the abomasum might provide a nearly perfectly linear slope, the intercept still depends on direct measurements of duodenal PB, which retain all of the inherent variability and potential inaccuracy. As discussed in the Appendix, because the intercept and slope are so highly correlated, errors in estimation of the intercept strongly influence the accuracy of the entire prediction equation. Paradoxically, this lack of precision and accuracy of measurement of duodenal PB (Broderick and Merchen, 1992; Stern et al., 1994; Firkins and Reynolds, 2005) is often touted as the main justification for the use of the urinary PD approach in the first place.

There are several prediction equations available for use, and accuracy of a predicted PB value is obviously dependent on appropriateness of the equation chosen. For example, in a recent evaluation study (González-

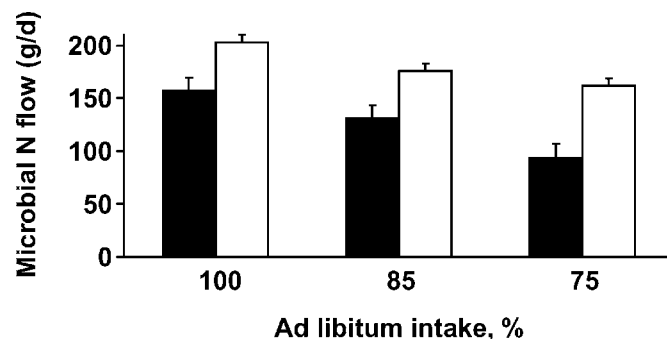


Figure 1. Microbial N flow measured using direct measurement of purine bases in duodenal digesta (filled bars) or predicted by measuring urinary excretion of purine derivatives (open bars). Means and SEM are averaged for values reported for liquid- and solids-associated bacteria for three different levels of feed intake. From González-Ronquillo et al. (2004).

Ronquillo et al., 2004), cows were in their fourth month of lactation, which would be intermediate between the 10- and 33-wk regressions from the calibration study (González-Ronquillo et al., 2003). Even though this late lactation equation seemed to overestimate duodenal PB flow (Figure 1), using the early lactation equation would have increased these estimates by approximately 25% more. Without knowing the actual PB flow in a future study in which it is not measured, more objective approaches are needed to choose which equation to use.

Besides the accuracy of equations for prediction purposes, this report illustrates another problem that is largely overlooked, and is potentially even more important. Declining DMI linearly decreased ($P < 0.05$) the predicted microbial N flow by about 20%, whereas there was only a linear trend ($P < 0.10$) associated with a 40% decrease in measured PB flow (Figure 1). González-Ronquillo et al. (2004) reasoned that the PD method had a greater sensitivity to detect treatment differences because the CV was approximately half that of the CV for direct measurement of PB, stating that their data “confirm the validity of the PD method as an alternative to conventional methods.” In contrast, the use of an algebraic equation ignores the error associated with the measurement of duodenal PB flow in the calibration study and primarily only contains the error associated with measurement of urinary PD in the new study, even though this error is (incorrectly) associated with the predicted duodenal PB flows that get analyzed statistically. The ramifications for this approach are demonstrated in the following two sections.

Microbial References. The error in predicting microbial N flow from urinary PD excretion can be further amplified by using a literature-based microbial reference standard. Predicted duodenal PB flow (Xnew) is converted to duodenal microbial N flow (MNpred)

$$\text{MNpred} = \text{Xnew}/(\text{PB:N}) \quad [4]$$

where PB:N is the ratio from a microbial standard that is harvested in such a way to represent the average of all of the microbes passing from the rumen. The variance of MNpred (which is a ratio of 2 random variables) is approximated (Gill, 1978) by this formula.

$$s_{\text{MNpred}}^2 = \frac{(\text{Xnew})^2(s_{\text{PB:N}}^2) + (\overline{\text{PB:N}})^2(s_{\text{Xnew}}^2)}{(\overline{\text{PB:N}})^4} \quad [5]$$

An approximated 95% confidence interval (CI) for the MNpred flow to the duodenum is given:

$$\text{MN}_{0.95} = \text{MNpred} \pm t_{(1-\alpha/2, n-2)} s_{\text{MNpred}} \quad [6]$$

Note that some literature estimates are expressed as purines, the actual component measured in the assay, whereas their values were likely to be expressed on an RNA basis, so we caution against using a literature-derived mean unless all components are based on known correct units. Moreover, we are not aware of any study in which PB (determined using HPLC) were expressed as a concentration of RNA (determined using the colorimetric assay). Because of disparity in estimates of the relative proportions of adenine and guanine (Ushida et al., 1985; Makkar and Becker, 1999), assuming a 1:1 ratio of bases, 50% PB of the total nucleotide bases in RNA, and accounting for the differences in molecular weights of bases and their respective nucleotides in RNA, we calculate that RNA should have approximately 22% PB. When using the purine assay for both duodenal samples and microbial standards, this constant for PB as a percentage of RNA is factored out, but it is needed when PB:N in [4] is based on a literature estimate using the colorimetric assay for purines that are expressed on a RNA basis. Moreover, those using the HPLC procedures should consider quantifying nucleic acid derivatives and not just the actual bases (Reynal et al., 2005).

Variance among PB:N in the literature probably results from the various laboratory deviations of the original procedure (Ushida et al., 1985; Aharoni and Tagari, 1991; Makkar and Becker, 1999) and even with combinations of these modifications (Reynal et al., 2003). In the first author's laboratory, reagents are made with tap water to increase pellet hardness, and we surmise that soft pellets might be a root cause for many of the modifications. Therefore, we performed a meta-analysis from individual RNA:N values from studies from that laboratory (Piwonka et al., 1994; Younker et al., 1998; Oldick and Firkins, 2000; Callison et al., 2001; Harvatine et al., 2002) all using the same procedure to demon-

strate how use of a common mean value should be avoided. In this case, laboratory quality control included evaluation of spiking and recovery and other steps to prevent artifact absorbance readings. All bacterial samples were from rumen samples that were blended to dislodge solids-associated bacteria (SAB) and composited over the feeding cycle to control for diurnal RNA:N. All were Latin square designs with a variety of treatments, with 2 and 3 studies using nonlactating and lactating cattle, respectively. In the model, lactation state, trial (lactation), period (trial \times lactation), and treatment (trial \times lactation) were all $P < 0.02$; cow (trial \times lactation) was $P = 0.12$ but was maintained as a random effect using Proc Mixed of SAS (SAS Institute, Inc.). The RNA:N ratio for nonlactating cows (1.136, SEM = 0.014) was greater than that for lactating cows (1.075, SEM = 0.010), and the overall mean (adjusted to the average effect of all other variables in the model) was 1.105 (SE = 0.009). From that final model, the RMSE was 0.0679. In contrast, when an overall average was obtained without accounting for any of these effects, the overall mean was 1.069, which is slightly different than 1.105 because of unequal numbers of cows and adjustment for other effects in the previous model. The RMSE was 0.2089, which is about 3-fold higher. Assuming errors in the new study to be independent from the errors in the calibration study, the SE (square root of [14] in the Appendix) obtained using the mean of Xnew from the new study should be added to the SE determined using each Xnew data calculated using an algebraic regression equation of the calibration study, as documented in the subsequent section.

Estimating the Actual Standard Error from using Prediction Equations. From [14] in the Appendix, the estimated variance of the mean predicted duodenal PB flow (290 mmol/d), which is the mean from the evaluation study (González-Ronquillo et al., 2004), is shown in Figure 2 based on the prediction equations for cows in early or late lactation from the calibration study (González-Ronquillo et al., 2003). In this scenario, the authors reported flows of microbial N calculated using either liquid-associated bacteria (LAB) or SAB. For simplicity of presentation, we averaged values for LAB and SAB (1.71 μmol of PB/g of N) and averaged the mean and standard error of microbial N flows that were calculated using either bacterial standard (i.e., assuming equal contribution of LAB and SAB to the duodenal bacterial N) in Figure 2. The added error from the prediction equation is a result of the large error associated with the late lactation prediction equation. When using [14] in the Appendix, we conclude that, in contrast with the authors' conclusions that the use of urinary PD to predict duodenal PB flow had about half

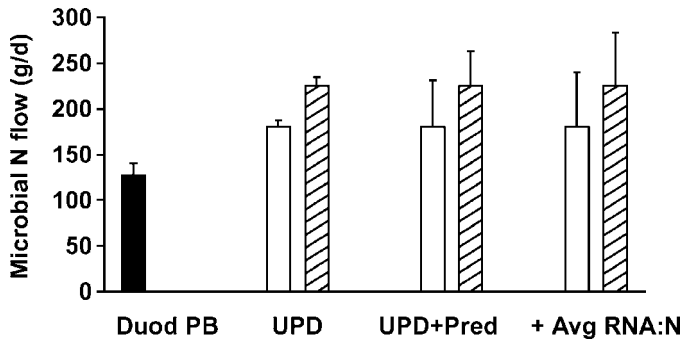


Figure 2. Microbial N reported in González-Ronquillo et al. (2004) that was measured directly using duodenal purine bases (Duod PB; solid bar) or microbial N that was calculated using prediction equations from late (open bars) or early (hatched bars) equations from González-Ronquillo et al. (2003). For the bars determined using urinary purine derivatives (UPD), the microbial N flow and SEM in the open bar are from Figure 1 but averaged over all DMI. Using the same mean UPD (290 mmol/d) from that report (González-Ronquillo et al., 2004), microbial N was calculated using the early lactation equation (first hatched bar from the left). The SEM was calculated by multiplying the mean SEM for the late lactation equation (associated with the open bar) by the difference in the means to obtain a projected SEM for the hatched bar. The SEM associated with prediction (equation [14]) was calculated using the late or early lactation regressions and added to that from UPD bars to estimate SEM in the predicted means (UPD + Pred bars). Finally, the SEM was calculated using equation [5] for bacterial RNA:N from different studies (Piwonka et al., 1994; Younger et al., 1998; Oldick and Firkins, 2000; Callison et al., 2001; Harvatine et al., 2002), assuming proportionality of means and SE for conversion to PB:N. This SEM was added to the SEM from respective bars labeled UPD + Pred to obtain the SEM for bars labeled + Avg RNA:N (sixth and seventh bars from the left).

the CV compared with direct determination of duodenal PB flow (and therefore more sensitive for treatment differences), the CV were actually >3-fold higher for predicted PB flow than for directly measured PB flow after the error of the original prediction equation was added (UPD + Pred bars in Figure 2). Using our RNA:N of 1.105 and RMSE of 0.0679 only slightly amplified error (by about 4%; data not shown), so the variation associated with a within-trial measurement of RNA:N or PB:N of a microbial standard, compared with its actual (unknown) mean, should be negligible. However, when using the unadjusted mean of 1.069 (RMSE of 0.2089), which would be necessary for any new trial, the CV was amplified >1-fold further (+ Avg. RNA:N bars in Figure 2), demonstrating clearly why researchers should harvest microbial samples for every study.

Improved Prediction of Duodenal Flow of Purine Bases. We suggest that a prediction equation derived from multiple studies with different animals and dietary conditions should be more robust for prediction of future studies compared with an equation derived from a single study. Moreover, as explained in the Appendix, inverse regression should be avoided to prevent error amplification (or infinity error). Therefore, a data

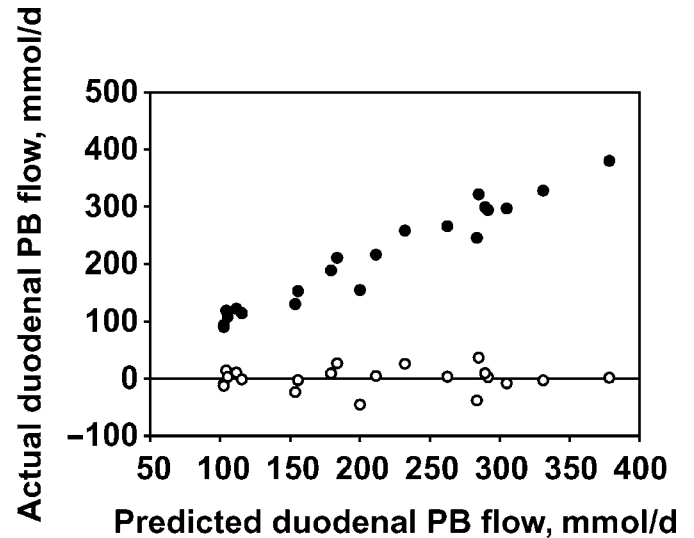


Figure 3. Observed (●) and residuals (observed – predicted, ○) for duodenal flow of purine bases (PB) predicted from urinary allantoin excretion corresponding with equation [7]. Data are adjusted for the random effect of experiment and are from different studies using similar procedures (Martín-Orúe et al., 2000; Orellano Boero et al., 2001; González-Ronquillo et al., 2003, 2004).

set was derived from 4 studies (see Figure 3 for references). Using the basic approach and variables described by Hristov et al. (2004b), backward elimination regression removed most of the variables because of lack of significance or because of high collinearity with remaining variables. With a limited data set in which duodenal PB and urinary allantoin excretion (both units are mmol/d) were both available, and including experiment as a random effect, the resulting improved equation was derived

$$\text{Duodenal PB flow} = 17.0 \text{ (SE} = 26.5\text{)} \quad [7] \\ + 1.037 \text{ (SE} = 0.090\text{)} \times \text{urinary allantoin}$$

The RMSE was 21.7, documenting a good fit (Figure 3). However, the data were derived from similar studies, which might have consistent sources of error, and no other terms remained in the model to help account for differences in endogenous contributions of PD that might differ across more diverse feeding situations.

Another data set was created in which urinary allantoin was measured even if duodenal PB flow was not. There were 339 observations from 30 references using cattle (see Figure 4 for details). Sheep data were omitted because sheep probably metabolize PB differently than do cattle (Shingfield, 2000). For this data set, the effect of lactation was significant, so 2 separate regressions were derived to predict urinary allantoin. For lactating cattle:

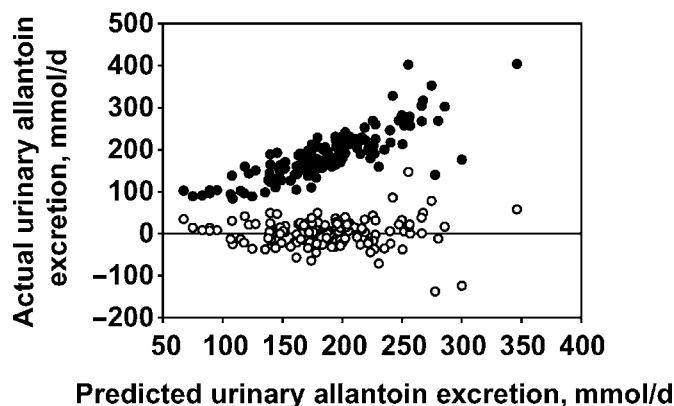


Figure 4. Observed (●) and residuals (observed – predicted, ○) for urinary allantoin excretion predicted from equation [8]. Data are adjusted for the random effect of experiment and are from V. Vulpe, B. Stefanon, and L. Gruber (personal communication) using their unpublished data and data from various published studies using cattle.

$$\begin{aligned} \text{Urinary allantoin (mmol/d)} &= 10.8 \\ &+ (3.07)(\text{milk yield, kg/d}) \\ &+ (29.5)(\text{RDP intake, kg/d}) + (16.9)(\text{NFC intake, kg/d}) \end{aligned} \quad [8]$$

where NFC was calculated as (100% – percentages of NDF, CP, ether extract, and ash) and SE were 11.8, 0.86, 7.30, and 2.61, respectively, and RMSE was 34.0. Two models were evaluated: either 1) DMI and nutrients as percentages of DM, or 2) intakes of all nutrients (percentage data \times DMI) but excluding DMI. In most cases, the former method should be considered first to avoid potentially high collinearity associated with variables remaining (a DMI component is in all of the terms in the latter approach). However, [8] was chosen based on Bayesian information criterion, acceptable residual structure (Figure 4), and variance inflation factor <10 , as described (Hristov et al., 2004b). In [8], all terms except the intercept were $P < 0.01$. Dry matter intake is the most important driving variable affecting MPS prediction (Oldick et al., 1999), and RDP and NFC percentages (the other components of RDP intake and NFC intake) would also be expected to be positively related to MPS (see earlier discussion). Milk production was highly significant in this model either because of 1) concurrent indirect associations with DMI that are not explained by RDP or NFC intake terms and therefore MPS in the rumen, or 2) associations with milk production and varying postabsorptive PB metabolism.

For nonlactating cattle,

$$\begin{aligned} \text{Urinary allantoin (mmol/d)} &= 28.9 \\ &+ (108)(\text{RDP intake, kg/d}) \end{aligned} \quad [9]$$

where SE were 12.5 and 19, respectively, and RMSE was 21.4. The residuals analysis (data not shown) documented no linear or mean bias. The intercept and RDP coefficients were $P < 0.10$ and $P < 0.01$, respectively. Continuous variables such as those in [8] and [9] should improve the robustness of future equations predicting duodenal flow of PB.

Latent Variables and Other Regression Issues. The flow of microbial N is a direct function of RNA:N ratio (i.e., microbial N = RNA flow to the duodenum/microbial RNA:N). If an assay has a constant but incomplete recovery of RNA, then both RNA flow and RNA concentration in the microbial reference could have the same factor for recovery, negating this error in microbial N flow. Therefore, the duodenal samples and microbial references should be performed in the same laboratory run. However, there are situations in which the errors for determination of RNA:N in microbes are independent of the errors in measurement of duodenal RNA (e.g., compounds might interfere with the assay for microbial samples but are more dilute and not problematic in duodenal samples). Such a problem can promote spurious relationships when one variable (RNA:N) is a latent variable in the calculation of another (microbial N).

The data for bacterial RNA:N from the same 5 trials described previously were adjusted for the effect of trial (St-Pierre, 2001a); the model would not converge when other effects (period or treatment within trial and lactation state) were included:

$$\begin{aligned} \text{Adjusted RNA:N} &= 1.24 \text{ (SE = 0.08)} \\ &- 0.00078 \text{ (SE = 0.00024) Microbial N (g/d)} \end{aligned}$$

In this model, bacterial RNA:N was inversely related ($P = 0.002$) to microbial N flow. Because the RNA:N value is a latent component of the microbial N calculation, the errors are not random. If any single RNA:N point is overestimated from its true value, the corresponding microbial N flow must be underestimated by the same degree; and if any RNA:N is underestimated, the microbial N must be overestimated. The regression is biased by the degree to which these points deviate from their true values (i.e., their errors are correlated). We strongly recommend that researchers should evaluate the correlation in the calculations used before publishing more potentially inaccurate inferences among variables.

As researchers perform more regression analyses, it is important to provide RMSE and the individual SE associated with regression coefficients to assess variation in the regression. Moreover, we note too many cases published in the literature in which a regression coefficient has a single significant digit. This is particularly

troublesome for polynomial regressions in which a quadratic or cubic term has a very low value (e.g., 0.0001), which when multiplied by a large number for the X^2 or X^3 term, can lead to up to 50% error simply due to over-rounding. This problem can easily be overcome by adding a second or third significant digit (the number of decimal places is irrelevant for rounding decisions) or else by decreasing the value of X (e.g., converting grams to kilograms).

Which Microbial Marker and Reference? A marker that is less variable among microbial populations would decrease the error from harvesting microbial standards that deviate from the true weighted mean of microbial populations, and ^{15}N enrichment varies less among microbial standards than do PB:N ratios (Broderick and Merchen, 1992). Although some dietary purines might escape ruminal degradation, the flow of bacterial N calculated using PB typically is similar to, or lower than, that calculated using ^{15}N (Hristov et al., 2005a). The authors suggested that the discrepancy could be a result of using PB:N ratios of samples collected from the rumen that did not correctly represent the PB:N of microbial matter (including that from lysed cells) flowing out of the rumen (see next section). However, the ^{15}N is assumed to be distributed throughout the entire bacterial cell, including proteins, nucleic acids, and cell wall fractions, in nearly identical enrichments. In the same way that diaminopimelic acid might overestimate bacterial N flow because of duodenal flow of ghost cell wall fractions (Stern et al., 1994; Volden, 1999), it should be determined if differential enrichment of ^{15}N from bacterial cell walls is biasing flows of omasal or duodenal N. However, such a scenario might be counterbalanced because ^{15}N infusion might form a gradient, with a higher ^{15}N enrichment at sites closer to the infusion and sampling sites (both through the rumen cannula) and a lower enrichment in the more distal sites of the rumen, which might underestimate microbial N flow. The gradient problem can be circumvented if markers are mixed with evacuated rumen contents outside the rumen before sampling (Hristov et al., 2004a).

Although feed particles provide the majority of substrate for bacteria, faster fluid dilution rate could increase the outflow of LAB compared with SAB, so duodenal bacteria should have more LAB compared with the rumen (Hristov and Broderick, 1996; Ahvenjärvi et al., 2002). Yet, we note considerable discrepancy among studies regarding the relative contribution of LAB and SAB to duodenal bacterial N (Volden, 1999; Ahvenjärvi et al., 2002; Rodríguez et al., 2003). The difference among LAB and SAB can change with increasing passage rates (Rodríguez et al., 2000). Unfortunately, it is difficult to extract the majority of SAB without poten-

tially lysing them, so the usage of molecular-based techniques (Firkins and Yu, 2006) or composition of very specific fatty acid (FA) isomers (Vlaemink et al., 2005) might help corroborate true contributions of LAB and SAB to duodenal microbial N flow. Researchers should be aware that FA composition of bacteria can change with dietary composition (Firkins and Yu, 2006). The optimization of microbial N flow based on unique FA isomers from rumen microbial samples at least avoids the potential bias associated with differential AA profile in RUP compared with the native dietary protein when optimizing microbial AA flow from AA profiles of different microbial samples (Reynal et al., 2005). Moreover, if these types of optimization procedures increase in frequency in future studies, we recommend for researchers to harvest protozoa using a filtering procedure rather than the traditional sedimentation technique, from which protozoal samples are highly contaminated with bacteria (Sylvester et al., 2005). They also should consider the potential for formalin to influence measured AA composition (Reynal et al., 2003).

Omasal Sampling. This discrepancy over contribution of LAB vs. SAB to represent duodenal microbial flow might be resolved by collecting microbial references from sites other than the rumen. When collecting bacterial references from the omasum, duodenal flow of microbial N was intermediate between calculations using bacteria harvested from the rumen and those from the duodenum (Ahvenjärvi et al., 2000). The acidity of the abomasum completely lyses protozoa and might selectively lyse certain bacterial groups. Thus, omasal sampling might provide a more accurate representation of the bacterial populations leaving the rumen. Based on molecular evaluation of microbial populations, omasal samples appear representative of the same populations of bacteria and protozoa in the rumen (Karnati et al., 2005). Moreover, omasal flows appear to represent duodenal flows of nutrients, particularly if omasal samples are reconstituted mathematically to true digesta (Ahvenjärvi et al., 2003). Although a 3-way marker system might be needed if Yb is infused into the rumen, marking and washing to remove loosely bound Yb allowed seemingly accurate reconstitution with a 2-marker system (Noftsker et al., 2005). Omasal sampling allows calculation of flow of protozoal cells from the rumen so that protozoal flow (Ahvenjärvi et al., 2002) and net generation time (Firkins et al., 2005) can be determined. However, more attention is needed to determine if differences in nutrient flows between the omasum and duodenum (Ahvenjärvi et al., 2000) are real or artifacts of sampling (even with mathematical reconstitution).

Table 1. Microbial protein synthesis from mixed ruminal microbes provided varying NFC in vitro in different studies

Incubation conditions	Study							
	Strobel and Russell (1986)		Strobel and Russell (1986)		Hall and Herejk (2001)		Holtshausen (2004)	
Initial pH	6.7		5.5–5.9		~6.7		~6.7	
Hexose added, g/L	1.8		1.8		3.0–3.2		4.1–4.6	
N source ¹	NPN, BCFVA		NPN, BCFVA		NPN, CAH		NPN, PDC	
Substrate	NFC		NFC		NFC + NDF		NFC + NDF	
Substrate response	MPS ²	E _{MPS} ³	MPS	E _{MPS}	MPS	E _{MPS}	MPS	E _{MPS}
Sucrose	7.1	0.105	4.7	0.079	29.6	0.133	17.9	0.09
Starch	5.9	0.104	3.8	0.078	36.7	0.146	21.0	0.11
Pectin	6.0	0.094	1.9	0.062	27.1	0.099	25.0	0.17

¹BCVFA = Branched-chain VFA, CAH = casein acid hydrolysate, PDC = pancreatic digest of casein.

²Cumulative microbial protein synthesis (mg).

³Efficiency of microbial protein synthesis, in mg of protein/mg of available hexose. Available hexose was measured (sucrose) or predicted (pectin and starch) for Strobel and Russell (1986) whose sealed incubations lasted 10 h; available hexose was calculated as the amount added at the start of the incubation for Hall and Herejk (2001) and Holtshausen (2004), which had 1-way release valves and which lasted 24 h.

Mechanistic Factors Influencing Prediction of MPS

In general, empirical systems typically predict MPS as if the E_{MPS} was constant, whereas there are many mechanistic factors to the contrary. For example, the varied carbohydrates that comprise NFC promote different E_{MPS} under different conditions in vitro (Table 1). No difference was detected in MPS when purified sucrose, starch, or pectin was incubated with nonprotein N (Strobel and Russell, 1986). At a lower pH, E_{MPS} from all 3 substrates decreased, with pectin showing the greatest decline. In another study (Hall and Herejk, 2001), when both nonprotein N and amino N were included in the medium, cornstarch gave the greatest maximum MPS compared with sucrose and citrus pectin in incubations containing both NFC and isolated bermudagrass NDF, but E_{MPS} was lowest for pectin. In a similar study from the same laboratory (Holtshausen, 2004) in which pectin was prepared as a suspension rather than added as a dry solid, MPS tended to be greater for pectin as compared with sucrose and starch, which did not differ, but the E_{MPS} actually was greatest for pectin. Despite differences in incubation method, N source, and method of microbial protein measurement, E_{MPS} appeared to depend on adequacy of AA and pH. Ruminal microbes are stimulated by adequacy of various end-products of protein degradation (Wallace et al., 1997), and both lower pH or excessive carbohydrate availability relative to RDP supply can increase energy spilling reactions (Russell, 1998) that uncouple ATP availability with MPS.

Although synchronization of energy from degraded carbohydrate and availability of amino-N and ammonia from RDP is both logical and supported by numerous

in vitro experiments, benefits of this synchronization in vivo are equivocal and possibly even a result of confounding factors such as dietary composition changes (NRC, 2001; Bach et al., 2005; Hristov and Jouany, 2005). Clearly, the rumen is not in a steady state (as assumed by most models) and BUN cycling to the rumen or storage and depletion of intracellular polysaccharide in both bacteria and protozoa help overcome transient deficiencies in RDP or energy (Hristov and Jouany, 2005). Still, some consistent in vivo results deserve further research, as described subsequently.

Increased consumption of NFC can reduce ruminal ammonia concentrations (Royes et al., 2001), which follows if increased NFC supply led to greater MPS and concurrent demand for RDP. A more curious change is that cattle consuming diets containing glucose, fructose, and sucrose had lower ruminal concentrations of branched-chain VFA (BCVFA) than did cattle consuming diets containing more starch (Heldt et al., 1999; Sannes et al., 2002; Hristov et al., 2005c), even when ruminal concentrations of ammonia did not differ (Heldt et al., 1999; Hristov et al., 2005c). Theoretically, the ratio of BCFVA to ammonia should remain similar across diets differing in NFC complement and similar in protein sources unless 1) branched-chain AA are preferentially degraded or conserved, or 2) BCFVA are utilized for processes other than AA synthesis. The decrease in BCFVA concentration might have been due to their elongation to branched-chain FA by the sugar-utilizing microbes (Allison et al., 1962). The decrease in BCFVA has not been reported for lactose (DeFraen et al., 2004). Similarly, BCFVA concentration decreased with increasing rate of cornstarch availability and corresponded with increased MPS for the treatment with

the fastest rate of NFC degradation (Lykos et al., 1997). However, generalizations should not be made because substituting molasses for high-moisture corn had a minor effect on ruminal concentrations of BCVFA (Broderick and Radloff, 2004). Ruminal ammonia concentration was only decreased at the lowest level of molasses feeding in both studies. From a meta-analysis of numerous experiments, despite having the greatest apparent ruminal NSC and NDF digestibilities compared with other processed corn sources, cows fed high-moisture corn had the numerically lowest MPS (Firkins et al., 2001). Although this could be a result of a deficiency in RDP or from energy spilling reactions, decreased rate of passage (Oba and Allen, 2003) also could decrease E_{MPS} .

Supplemented NFC can depress fiber digestibility through effects of pH (sucrose, *in vivo*; Khalili and Huh-tanen, 1991), inhibitors produced by the microbes (glucose, *in vitro*; Piwonka and Firkins, 1996), and if RDP is limiting (glucose, fructose, sucrose, starch, *in vivo*; Heldt et al., 1999). In the latter case, it may be a matter of the NFC-utilizing microbes out-competing fiber utilizers for scarce nutrients (Jones et al., 1998). Fibrolytic bacteria also can be stimulated by preformed AA (Bach et al., 2005). However, there is evidence that NFC may not affect (starch) or may actually increase (glucose, fructose, sucrose) fiber digestibility if RDP is not limiting (*in vivo*, Heldt et al., 1999; continuous culture, Vallimont et al., 2004). It is hoped that emerging technology will help increase our understanding of these complex interactions so that models can be more robust over a wider variety of feeding situations.

Although many mechanistic models (Dijkstra et al., 1998a) prioritize structural and NSC as the major energy to support MPS, most *in vivo* studies still report E_{MPS} on an OM basis. The E_{MPS} calculations were more statistically revealing when expressed on carbohydrate rather than OM bases for studies replacing forage with cottonseed (Harvatine et al., 2002) or varying degree of unsaturation of fat sources (Oldick and Firkins, 2000). Glucose (Lou et al., 1997), fructose, sucrose, and fructan (Thomas, 1960) can be converted to α -glucan and stored as microbial glycogen by ruminal microorganisms. The significant flow of nondietary α -linked glucose (188 g/d) to the small intestine reported for steers consuming a fescue hay diet (Branco et al., 1999) likely came from conversion of dietary carbohydrates other than starch to microbial α -glucan. Also, although documented rates of disappearance for glucose, sucrose, and lactose are very high (Weisbjerg et al., 1998), the actual production of microbial products from these substrates may proceed at a slower rate as the stored carbohydrate becomes catabolized later during the feeding cycle.

VFA Metabolism in the Rumen

Importance for Integration of Ruminal Metabolism. The rate of ATP availability should be coupled with the ATP needs for optimal microbial growth or else the rate of VFA production becomes uncoupled with the amount of MPS. When RDP is adequate, carbohydrate availability determines MPS in the rumen (Hoover and Stokes, 1991) and efficiency of ammonia use (Hristov et al., 2005c). Microbial protein is the most important source of essential AA for the lactating dairy cow for milk protein synthesis, but the energy from VFA needs to be further synchronized with MP to improve the efficiency of conversion of dietary protein into milk protein. Based on a compilation of studies, changes in the supply of individual VFA clearly are related to microbial protein supply and therefore milk yield and composition (Thomas and Martin, 1988). Increases in supply of acetic acid are associated with increased milk yield and milk fat concentration; and butyric acid, with milk fat concentration. However, propionic acid supply was inversely related with milk fat concentration but positively related with milk protein concentration. These results are explained by the glucogenic or ketogenic nature of the infused VFA and repartitioning effects of the VFA through hormonal changes (Dijkstra, 1994). Besides potentially changing metabolism resulting from changes in amounts of precursors, increased propionate should promote insulin secretion (Brockman, 1993) and can regulate DMI by high-producing dairy cattle (Allen, 2000).

Increasing grain in the diet typically increases the acetate:propionate ratio, but the major response to changes in molar proportions results from changes in propionate production (Sutton et al., 2003). Although infusion of VFA reduces variation (a constant amount is infused) and can be used to estimate VFA production rate (France and Siddons, 1993; Martin et al., 2001), VFA infusion studies need to be put into perspective with potential effects on DMI (Allen, 2000) and the association of VFA proportions with biohydrogenation intermediates that occur simultaneously in noninfused animals with similar changes in VFA profile (see later discussion).

Composition and Rate of Degradation of Substrate. Numerous studies have demonstrated that the type of VFA produced in the rumen is related to the composition of the diet (Dijkstra, 1994; De Visser et al., 1998; Sutton et al., 2003). Stoichiometric parameters, which are estimated by analysis of a large number of *in vivo* observations, are provided in Table 2. Degradation of fiber compared with starch yielded higher amounts of acetic and lower amounts of propionic acid, as expected (see earlier discussion). In addition to chem-

Table 2. Stoichiometric parameters for fermentation of substrates degraded in the rumen for roughage diets and concentrate diets

Substrate	60% Roughage				40% Roughage			
	Ac	Pr	Bu	Vi	Ac	Pr	Bu	Vi
	(mol produced/mol of substrate degraded)							
Cellulose	1.32	0.17	0.23	0.03	1.58	0.12	0.06	0.09
Hemicellulose	1.13	0.36	0.21	0.05	1.12	0.51	0.11	0.07
Protein	0.40	0.13	0.08	0.33	0.36	0.16	0.08	0.33
Starch	1.19	0.28	0.20	0.06	0.80	0.60	0.20	0.10
Soluble CHO	1.38	0.41	0.10	0.00	0.90	0.42	0.30	0.04

¹Ac = Acetic acid, Pr = propionic acid, Bu = butyric acid, Vi = valeric acid, and CHO = carbohydrate. Adapted from Dijkstra (1994).

ical composition of substrate, other factors such as frequency of feeding or forage particle size also affect VFA concentration as well as production rates (Sutton, 1985). However, the majority of these studies were not done with cows with a high DMI or level of milk production.

High concentrations of substrate generally shift fermentation patterns from acetic acid to butyric and lactic acids in vivo. The latter two act as a sink that disposes of excess reducing power (Wolin et al., 1997). Nocek and Tamminga (1991) demonstrated that, within substrate type (NDF, starch, sugars, and protein), rates of degradation were highly variable. Total ruminal VFA concentration tended to be higher for high-moisture corn than for dry cracked corn diets, probably reflecting higher ruminal degradability (Krause et al., 2003). Ruminal acetate concentration tended to be lower and butyrate was lower, whereas ruminal propionate concentration increased, when the dry cracked corn was replaced by high-moisture corn. In another study (Lykos et al., 1997), as rate of NSC degradation of corn grain increased in the diet, propionate concentration increased linearly. In two studies (Broderick and Radloff, 2004) evaluating replacement of high-moisture corn with molasses, however, concentrations of VFA were affected minimally, perhaps because ruminal NSC digestibility of high-moisture corn would be expected to be high already (Firkins et al., 2001).

VFA Profiles During the Transition Period. A study was conducted by Dann et al. (1999) to evaluate the response of cows pre- and postpartum to changes in rate of rumen-degradable starch. There was no difference in VFA profile when steam-flaked corn replaced dry cracked corn in the prepartum diet (DMI averaged 14 kg/d of a 34% concentrate diet). However, total VFA and propionate concentrations increased and BCVFA decreased when steam-flaked corn replaced corn in the ration in the postpartum period (20 kg/d DMI of a 49% concentrate diet). In a similar report by Markantonatos et al. (2004), ruminal VFA concentrations and pool sizes

were measured in cows pre- and postpartum. Molar proportions of ruminal acetate and isoacids were lower, whereas propionate and valerate were higher, than in prepartum cows. However, pool size and net production was higher for all VFA postpartum compared with ruminal VFA proportions and pool sizes prepartum. Because the amount of fermentable starch varies according to DMI and concentrate level or source, more information is needed to quantify and predict supply of VFA during this critical period when the amount of fermentable substrate changes so rapidly.

VFA Concentration vs. Production Rates. Concentrations and molar proportions of VFA in the rumen are commonly measured based upon the assumption that relative concentrations represent either VFA production, absorption, or both (Owens and Goetsch, 1988). Concentration of an acid in the rumen is a balance between the rate of production and the rate of removal or interconversion from the pool. Moreover, concentrations can vary with sampling time and sampling site. Near the cardia, for example, water and saliva entering the rumen can dilute VFA concentrations without altering their relative proportions (Owens and Goetsch, 1988).

In a detailed report comparing various factors related to VFA production (Sutton et al., 2003), lactating cows were fed either a high concentrate diet (10:90 forage to concentrate) or a normal diet (40:60 forage to concentrate; forage was mature ryegrass, so forage NDF should have been well above minimum guidelines established by the NRC, 2001). Pooled across both diets, the slopes of linear regression between concentration of VFA and production rate differed among the VFA. The relationship changed when molar proportions of net production rates were compared with molar proportions of concentrations. Only propionate production rate was related well to both its concentration and molar proportion. The researchers suggested that the common representation of data as molar proportions of concentrations was acceptable for acetate and propionate

but potentially misrepresentative for butyrate. Although these relationships were not different among diets, France and Siddons (1993) suggested that relative concentrations of the individual FA might be a more reliable index of the relative VFA production rates with forage diets but would appear less reliable with higher concentrate diets.

Interconversions Between VFA in the Rumen.

Considerable amounts of carbon exchange between ruminal VFA and therefore may be important in interpretation of VFA concentration data. According to Bergman (1990), production rate is more biologically meaningful when it is corrected for exchange ("net production rate" or "effective production rate"), particularly when these data are integrated with whole-body metabolism. Moreover, we note that researchers need to consider the site of the carbon label for such future studies and possibly for interpretation of previous reports (Sutton, 1985; Martin et al., 2001).

Many research groups utilized data from isotope dilution studies involving either single or simultaneous intraruminal infusions of ^{14}C -labeled VFA to estimate net production rates that take into account 1) the molar proportions of VFA produced in the rumen, 2) the ^{14}C recovered in VFA pools, and 3) the stoichiometry of VFA (Leng and Leonard, 1965; Leng et al., 1968). The consistent finding among these studies is that there is extensive interconversion between acetate and butyrate in the rumen. In a number of studies with cattle (Esdale et al., 1968; Sharp et al., 1982; Sutton et al., 2003), 55% of butyrate (range: 17 to 84.5%) was in equilibrium with 13% of acetate (range: 8.7 to 18%). However, Bergman (1990) and Brockman (1993) reported that these numbers could increase to 80 and 20% for the equilibrium between butyrate and acetate, respectively. Sutton et al. (2003) reported a transfer of carbon from butyrate to propionate at the levels of 13.4 and 10.4% of the total propionate production in the rumen of lactating cows fed either normal or low roughage diets, respectively. However, only 1.6 and 4% of the butyrate came from propionate, respectively.

Intraruminal Metabolism of VFA. One way to estimate VFA production rate is by measuring the amounts of VFA appearing in the blood stream (Bergman, 1990). This is done by multiplying the rate of portal blood flow by the concentration differences of individual VFA between arterial and portal blood. Due to the different extent of VFA metabolism by the ruminal mucosa during the absorption process, the appearance of VFA in the portal blood is not identical to VFA production in the rumen (Sutton et al., 2003). Furthermore, appearance of VFA in the portal blood also includes VFA produced in the cecum and colon (Bergman, 1990). Sehested et al. (1999) reported a net production of acetate

within bovine ruminal epithelium, and Kristensen et al. (2000a,b) found a minimal intraepithelial metabolism of acetate in sheep fed grass hay. With regard to propionate metabolism within the ruminal epithelial cells, only 5 to 15% of propionate was metabolized, and the rest was recovered in the portal blood. Weigand et al. (1972) also found minimal degradation of propionate in isolated ruminal papillae of steers.

A considerable amount of VFA are metabolized in the portal-drained viscera in cattle (Baird et al., 1974; Reynolds and Huntington, 1988). Peters et al. (1990b) showed that up to 87% of the intraruminally produced acetate disappeared from the reticulorumen of beef steers fed a mixed diet, whereas the remainder passed into the lower digestive tract. Intraruminal disappearance of propionate was found to range between 40 and 66% of its production or infusion rates (Peters et al., 1990a,b). Propionate metabolism by the rumen epithelium gives rise to L-lactate, CO_2 (Harmon et al., 1991), and probably alanine and glutamate (Gäbel et al., 2002). However, propionate conversion to lactate seems to be insignificant in cattle. Weigand et al. (1972) reported that only 2.3% of the propionate absorbed across the rumen epithelium was converted to lactate. Bergman (1990) indicated that only 3 to 5% of the propionate would be expected to be metabolized to lactate in the rumen epithelium. The majority of lactate present in ruminal epithelial cells probably derives from glycolysis of glucose coming from arterial sources (Gäbel et al., 2002). In studies using washed and isolated reticulorumens, Gäbel et al. (2002) reported that the irreversible loss rate of n-butyrate was 1.1 to 1.8 times that of propionate. Furthermore, Kristensen and Harmon (2004a,b) have shown that portal recovery of butyrate varied between 27 and 54%, depending on the absorption rate of butyrate through washed reticulorumens of steers. Kristensen and Harmon (2004a) concluded that the capacity of the ruminal epithelium to metabolize butyrate is limited. If butyrate absorption exceeds the metabolic capacity, it affects rumen epithelial nutrient metabolism and therefore nutrient capacity to the liver and peripheral tissues. The capacity of isolated ruminal epithelial cells of sheep to oxidize substrates was largely unaffected by ME intake or dietary forage-to-concentrate ratio in the diet (Baldwin and McLeod, 2000). Improved efficiency of metabolism by ruminal microbes should, by extension, help increase the efficiency of metabolism in the mammary gland and other peripheral tissues. Improved quantification of VFA production is therefore important for integrative metabolism in the rumen with that in the entire cow's body (Baldwin et al., 1994).

Monensin, Ruminal FA Metabolism, and Milk Composition

Interactions with Dietary Fat and Monensin.

Milk protein depression (lower protein percentage but not necessarily protein production) has long been associated with the feeding of supplemental dietary fat to dairy cows (DePeters and Cant, 1992; Wu and Huber, 1994) and has been given considerable attention by members of NC-1009. There is a similar response in VFA metabolism resulting from supplementing monensin and potential interactions of monensin with dietary fat (McGuffey et al., 2001). Because interactions of monensin with forage particle size and feeding method on milk fat percentage have been detected (Duffield et al., 2003), microbial population changes probably help explain some of the interactions and inconsistencies. Moreover, insulin responses could be influencing metabolism resulting from feeding monensin (McGuffey et al., 2001) or supplemental fat (Griinari et al., 1997). Reviews (McGuffey et al., 2001; Tedeschi et al., 2003; Ipharraguerre and Clark, 2003) provide more extensive information beyond our scope to emphasize future research needs for the high-producing dairy cow.

Monensin Effects on VFA Profile and Milk Production and Composition. There are few studies examining effects of monensin on VFA production rates and their kinetic parameters in the lactating dairy cow. Armentano and Young (1983) demonstrated that monensin increased ruminal propionate production at the expense of ruminal acetate production on a 70% hay diet fed to steers. They also showed that there was a negligible exchange of carbon atoms between propionate and butyrate in the rumen. Propionate production rate increased by 30% when steers were fed monensin in a high-concentrate diet (Van Maanen et al., 1978). Although long-term responses deserve further study, persistence of this response was projected by McGuffey et al. (2001).

Monensin generally is recognized to increase milk and milk protein production moderately (McGuffey et al., 2001). However, any monensin effect on lactation performance might be related to time with respect to parturition (Ipharraguerre and Clark, 2003). Markantonatos et al. (2004) estimated VFA kinetics in transition dairy cows supplemented with monensin (Table 3). There was no effect of monensin on production rates or interconversions of VFA; however, DMI (pre- vs. postpartum) effects on all VFA kinetic measures were significantly different. Because increased efficiency of glucose metabolism from propionate should spare AA for protein synthesis and feeding monensin could alter the AA composition of MP (by increasing escape of dietary protein), further research is needed to understand more

accurately the effects and interactions of monensin on glucose metabolism and milk protein synthesis in the dairy cow.

Biohydrogenation Intermediates. Monensin can depress milk fat concentration (McGuffey et al., 2001), probably through changes mediated by biohydrogenation intermediates (Jenkins et al., 2003). Historically, much knowledge was gained by studying the enzymatic activities by pure cultures of *Butyrivibrio fibrisolvens*, an important part of group 'A' bacteria that isomerize unsaturated FA to 18:2 *cis*-9, *trans*-11 and later biohydrogenate them to *trans*-11 18:1 isomers (Harfoot and Hazlewood, 1997). However, more recent evidence has documented that other groups of competing group 'A' bacteria, particularly *Megasphaera elsdenii*, might convert 18:2 to *trans*-10 intermediates (Wallace et al., 2005) and could be responsible for depressed milk fat synthesis (Kim et al., 2002). The rate-limiting step for complete biohydrogenation is the reaction catalyzed by group 'B' microorganisms wherein the 18:1 *trans* isomers are completely reduced to saturated stearic acid (18:0). Although these bacteria are sensitive to low pH, as would occur with increased grain feeding (Qiu et al., 2004), functional description of the group 'B' bacteria has been severely limited by the lack of cultured representatives. Using molecular approaches, they now appear to be specific groups of *Butyrivibrio hungatei* (van de Vossenberg and Joblin, 2003) or *Clostridium proteoclasticum* (Reilly and Attwood, 1998), as discussed by Wallace et al. (2005).

Most of the group 'A' bacteria degrade starch or fiber or both. In the *in vitro* study of Jenkins et al. (2003), both addition of soybean oil and monensin promoted *trans*-10 18:1 accumulation. However, the response was only additive when barley was used as substrate (compared with corn). The authors related starch availability to a shift in group 'A' bacteria. In the study of Looor et al. (2004) with lactating cows, supplemental linseed oil tended to be biohydrogenated through the milk fat-depressing *trans*-10 18:1 pathway to a greater extent when the oil was fed in a higher concentrate diet than in a higher forage diet.

Because *M. elsdenii* is insensitive to physiological doses of monensin (Rychlik et al., 2002), but *B. fibrisolvens* is inhibited (Nagaraja et al., 1997; Callaway et al., 1999), monensin addition to dairy rations might increase fluxing of unsaturated FA through *trans*-10 intermediates of 18-carbon FA. The former bacterium has been shown to increase with increased grain feeding while the latter decreased, presumably because of increased tolerance by *M. elsdenii* to low pH and perhaps to increased availability of lactate as a substrate (Klieve et al., 2003). Another predominant bacterium, *Selenomonas ruminantium*, is insensitive to monensin (Cal-

Table 3. Ruminal VFA parameters estimated from cows fed a control or monensin (M) diet in the prepartum and postpartum periods

Parameter estimate	Prepartum			Postpartum		
	Control	M	SE	Control	M	SE
AcIBu, ¹ mol/d	1.90	1.93	0.45	3.84	2.92	0.55
VFA pools, mol						
Acetate	2.117	1.895	0.121	2.538	3.368	0.698
Propionate	0.885	0.991	0.132	1.569	1.722	0.236
Butyrate	0.650	0.512	0.128	0.762	0.766	0.120
Total VFA production, mol/d						
Acetate	24.2	22.6	2.0	35.9	34.1	5.5
Propionate	8.9	12.2	1.9	19.7	20.6	1.5
Butyrate	10.5	6.9	1.9	11.5	11.7	1.4
Net VFA production rates, mol/d						
Acetate	15.6	18.6	3.0	28.2	28.1	5.2
Butyrate	6.8	5.0	1.2	7.6	8.6	1.2

¹AcIBu = Acetate incorporation rate into butyrate pool. From Markantonatos et al. (2004).

laway et al., 1999). Although some strains consume lactate, others make it as a principal fermentation end-product (Stewart et al., 1997). Therefore, it could compete with *M. elsdenii* when NSC availability in the rumen increases (Brossard et al., 2004). In the latter study, entodiniomorphid protozoa were credited with increased metabolism of lactate to help prevent lactic acidosis. Isotrichid protozoa are more insensitive to monensin than are the entodiniomorphids, although adaptation by entodiniomorphids in vivo seems to occur (Towne et al., 1990).

Protein Degradability. *Butyrivibrio fibrisolvens* and *Prevotella ruminicola* are among the most proteolytic bacteria in the rumen (Stewart et al., 1997), but most strains are very sensitive to monensin (Callaway et al., 1999; Callaway and Russell, 2000), whereas *M. elsdenii* is a potent deaminating bacterium that is insensitive to monensin (Rychlik et al., 2002). Furthermore, some strains of deaminating bacteria ("hyperammonia producers") might adapt to monensin (Callaway et al., 1999) but also might be very sensitive to fat (McIntosh et al., 2003). Therefore, future research is needed to evaluate potential changes in protein supplementation strategy with and without monensin, particularly in diets with supplemental fat.

Protozoal Metabolism. Protozoa are credited with contributing 40 to 50% (Bach et al., 2005; Hristov and Jouany, 2005) of the total microbial biomass in the rumen. However, this contribution is modeled to decrease with decreasing ruminal pH and increasing passage rate for dairy cows (Dijkstra et al., 1998b). In dairy cattle consuming 24.5 kg/d of DM, protozoal N was estimated to contribute 13% or less of total microbial N in both ruminal and duodenal samples (Sylvester et al., 2005). The contribution of protozoal protein to MP under different dietary conditions is not well known,

and more work is needed for high-producing cows. However, the effects of protozoa on ruminal N metabolism are much better known because of the large amount of defaunation studies available in the literature (Hristov and Jouany, 2005), mostly with nonlactating or low-producing animals. Defaunation protocols seem impractical in production scenarios for the near future. Suppression of protozoal numbers is more feasible and likely will increase E_{MPS} without the negative impact of complete defaunation (e.g., depressed fiber digestibility).

Among the antiprotozoal agents, unsaturated long-chain FA and saturated medium-chain FA are promising candidates (Hristov and Jouany, 2005). However, these FA modify other microbial populations (Firkins and Yu, 2006), including methanogenic archaea and various types of bacteria. Despite decreased protozoa-mediated intraruminal N recycling and increased E_{MPS} from feeding fat (Firkins, 1996), more mechanistic parameterization of this effect is needed (Baldwin et al., 1994).

CONCLUSIONS

There are many potential interactions of dietary conditions on bacterial populations and resultant availability of metabolites affecting the amount of MPS and milk protein synthesis. With the increasing reliance on meta-analyses for either direct calibration of prediction equations or for evaluation of mechanistic predictions, researchers need to be aware of statistical pitfalls embedded with these approaches to ensure that quantitative estimates are accurate.

Advancing progress requires a better understanding of the contribution of protozoa, LAB, and SAB to microbial protein both produced in the rumen and actually

flowing to the duodenum. Concomitantly, improved procedures for evaluating specific populations of microbes and VFA production and interconversions using emerging technology should help explain previously unexplained variation and therefore improve integration with postabsorptive metabolism in the high-producing dairy cow. Investigation of the effects and interactions of specific carbohydrates and RDP end-products on MPS and the partitioning of feed carbohydrates could help to better explain results within and among studies. Improved feed and chemical description of diets would allow a more coherent, informed picture to be drawn from the diverse studies performed.

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APPENDIX

Confidence Intervals and Errors from Inverse Regression

For the following derivation, let X = duodenal PB flow (mmol/d), Y = urinary PD excretion (mmol/d), and \bar{X} = mean duodenal PB flow in the calibration data. The regression of Y on X can be obtained from the model

$$Y = \beta_0 + \beta_1 X + \varepsilon \quad [10]$$

where β_i represent the true (population) regression coefficients, and ε is the vector of errors, which have a mean of zero and a variance of σ_e^2 . Each ε_i represents the deviation of each observation from the true regression with known parameters β_0 and β_1 . Because these parameters are not known, b_0 and b_1 are estimated from measurements of X and Y from a sample using regression techniques (i.e., least squares).

After simulating the data from a comparable study (Martín-Orúe et al., 2000) from which all data were depicted and corroborating that the regression was virtually identical to that reported, the variance-covariance matrix showed that the correlation between b_0 and b_1 was -0.95 . This means that an underestimation

of b_0 will result in an almost inversely proportional overestimation of b_1 compared with their true values from [10], and vice versa. We assumed a similar negative correlation in the calibration data of González-Ronquillo et al. (2003). One could argue that these errors affect all treatments in the new study to the same degree, so the rankings of treatments would be similar. However, the real problem is that these biases cause the true error to be greater than expected, so the ability to draw correct statistical inferences of treatment difference is negatively impacted.

If the regression were taken from a population (as in [10]), the variance (σ_e^2) would be the same at each X , Y coordinate, and flanking parallel lines would depict the regression's CI. However, because it is taken from a sample, the error actually varies with each X , Y coordinate. The variance of Y_{pred} at a given X (X_i) is estimated as

$$s_{Y_{\text{pred}}}^2 = \text{MSE} \left[1 + \frac{1}{n} + \frac{(X_i - \bar{X})^2}{\sum (X_i - \bar{X})^2} \right] \quad [11]$$

where MSE is the mean square error, which is an unbiased estimate of σ_e^2 . A 95% CI for Y_{pred} can be obtained

$$Y_{0.95} = Y_{\text{pred}} \pm t_{(1-\alpha/2, n-2)} s_{Y_{\text{pred}}} \quad [12]$$

where $t_{(1-\alpha/2, n-2)}$ is from the t distribution with $n-2$ degrees of freedom.

Using the data provided by González-Ronquillo et al. (2003), we estimated the variance at each point and produced a 95% CI for cows in early lactation (Figure 5a). We point out that, because b_0 and b_1 are only estimates of β_0 and β_1 , the CI flares as the data diverge from the mean; moreover, the greater the correlation between b_0 and b_1 , the greater the flaring.

The variation transferring from the sample calibration data to the prediction in the new study is further exacerbated when one is estimating X from Y (Draper and Smith, 1998). When performing an inverse regression, [10] is rearranged to solve for X after b_0 and b_1 have been estimated from a sample. The intent is to produce a prediction of X (X_{new}) for observations made using new measurements (Y_{new}) from a new experiment (different from the calibration study).

$$X_{\text{new}} = \left[\frac{Y_{\text{new}} - b_0 - e}{b_1} \right] \quad [13]$$

In [13], b_0 and b_1 are not independent (Draper and Smith, 1998) and, as described previously, are highly negatively correlated. Although typically ignored, the

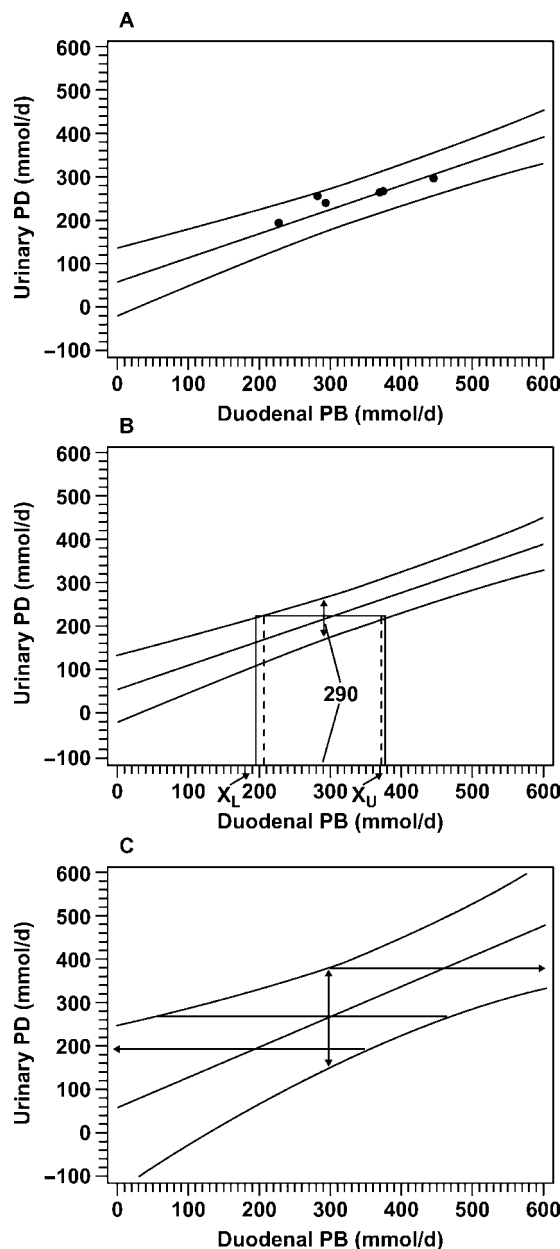


Figure 5. Regression from González-Ronquillo et al. (2003) and confidence interval (CI) calculated from equation [12] for cows in early lactation (panel A). For panel B, at the mean (290 mmol/d) predicted duodenal flow of purine bases (PB) from another study (González-Ronquillo et al., 2004), a double arrow depicts the CI for the Y vs. X regression. The CI for the inverse regression (X vs. Y ; solid line) at 290 mmol/d of duodenal PB (about 220 mmol/d of urinary purine derivatives, PD) would be determined by spanning the CI at a line parallel to the horizontal axis and extrapolating the lower and upper (X_L and X_U) points (Draper and Smith, 1998). An approximated CI for that point can be calculated using equation [15] (X_L and X_U corresponding with the dashed lines). Panel C depicts the regression (González-Ronquillo et al., 2003) and its CI (calculated from [12]) for cows in late lactation. In this case, the asymmetric X vs. Y CI (solid line) compared with the Y vs. X CI (vertical double arrow) and the problems of unbounded X vs. Y CI (Draper and Smith, 1998), depicted with horizontal single arrows, become more pronounced than in panel b.

variance associated with using the calibration study to predict the X_{new} data from the new experiment is therefore calculated using the MSE, b_1 , n , X_i , and \bar{X} from the calibration study combined with the X_{new} from the new study (Draper and Smith, 1998)

$$s_{X_{\text{new}}}^2 = \frac{\text{MSE}}{(b_1)^2} \left[1 + \frac{1}{n} + \frac{(X_{\text{new}} - \bar{X})^2}{\sum (X_i - \bar{X})^2} \right] \quad [14]$$

An important distinction between [11] and [14] is that the latter has the $(b_1)^2$ term in the denominator. Equation [14] is neutral only if the 2 variables have the same scale and dispersion. Based on our typical interpretation of efficiency (a certain yield of product forming from its precursor), the origin of the current regression approach is understandable. However, inverse regression and which choice of Y and X is a lengthy and well described statistical question (Tan and Iglewicz, 1999) that will not be discussed further.

Although a more accurate CI can be calculated (Draper and Smith, 1998), this CI can be approximated using the square root of [14] as

$$X_{0.95} = X_{\text{new}} \pm t_{(1-\alpha/2, n-2)} s_{X_{\text{new}}} \quad [15]$$

Note that the approximated CI from [15], which is often called the fiducial limits in the literature, is symmetric with respect to X_{new} , whereas the true CI is not symmetric (Draper and Smith, 1998). From interpolation of Figure 5b, one can determine that the CI for X vs. Y is about 37% larger relative to its mean than is the CI for Y vs. X , and the difference magnifies as the X , Y coordinate deviates from the mean. As the X value deviates from the mean in either direction, eventually a point will be reached at which X_L and X_U will become unbounded and there is virtually an infinite number of possible predicted X values that will fall within the CI. This error amplification and unbounded CI for predicted X from inverse regression is magnified when evaluating the regression for cows in late lactation (Figure 5c), which had considerably more residual variation associated with the regression equation (and was the one chosen). Thus, the microbial N predicted from this algebraic equation, compared with duodenally derived data, would have had a much greater likelihood of predicting X below or above the limits at which an X vs. Y CI is unbounded and actually would have much worse precision than the original directly measured PB data, particularly as the values deviate more from the mean.